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Evaluation of thiosemicarbazones and semicarbazones as potential agents anti-*Trypanosoma cruzi*

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ABSTRACT

Synthetic thiosemicarbazones and semicarbazones were evaluated for their *Trypanosoma cruzi* trypomastigotes obtained from LLC-MK2 cell cultures. In general, thiosemicarbazone derivatives were most effective and among them the 4-N-(2′-methoxy styryl)-thiosemicarbazone was chosen, to compare the *in vitro* effect against amastigotes of *T. cruzi* lodged in mouse peritoneal and human macrophages. A potent trypanocidal effect was observed that was more pronounced against parasites internalized in human macrophages. A potential target for this compound was also evaluated by measuring the nitric oxide synthase activity through NADPH consumption. A significant decrease in enzyme activity was observed. In contrast to the cytotoxic effect observed with benznidazole, no macrophage toxicity was observed for any of the compounds, indicating that their activity was specific for the parasite forms investigated.

1. Introduction

Chagas' disease is a leading cause of heart disease in the Americas. At least 8 million people are infected with *Trypanosoma cruzi*, resulting in more than 14,000 deaths each year (DNDi, 2010). Morbidity is relatively high, 17–30% of chronic chagasic patients display variable clinical manifestations, including cardiomyopathy and pathological gastrointestinal dilation (Higuchi, 1995; Rezende, 1993). Chagas' disease is generally transmitted to humans via blood-sucking triatominea through an infectious metacyclic trypomastigote form of this protozoan parasite (WHO, 2011). These forms invade mammalian host cells where they change into the intracellular amastigote form and replicate, primarily in muscle cells, fibroblasts and macrophages. Amastigotes then transform back to trypomastigotes and, due to the rupture of the cells, the infectious forms are released into the bloodstream, reaching other cells and tissues and amplifying the infection (WHO, 2011). Treatment options with nifurtimox (Lampit™) and benznidazole (Radanil™, Rochagan™) for Chagas' disease are limited due to their limited effect towards different parasite isolates and disease phases and systemic toxicity, which leads to adverse effects (Maya et al., 2007; Prata, 2001; Soeiro et al., 2009). Currently, most anti-parasitic drugs are considered orphan drugs, with the main exception of antimalarials. The pharmaceutical considerations outweigh all others, because the economic return on the development of anti-parasitic drugs is limited. Therefore, it is necessary to find less expensive alternatives for the treatment of Chagas' disease (Cançado, 1997; Prata, 2001).

Thiosemicarbazones and semicarbazones are classes of compounds with medical potential due to their capacity to inhibit the growth of several pathogens (DoCampo, 1990). Those compounds have been show to have antiviral, antibacterial, antitumor and antimalarial activities (Dobek et al., 1980; Kalinowski et al., 2007; Smee and Sidwell, 2003). Furthermore, studies concerning their biological activity show that these compounds are active against *T. cruzi* among others trypanosomatids (Beraldo and Gambino, 2004; Fujii et al., 2005; Jeremy et al., 2008; Klayman et al., 1979).

Some authors hypothesize that the structural specificity of these compounds could have as targets intracellular components, such as the enzyme ribonucleotide reductase, which is essential for DNA synthesis and consequently for cellular division, or their ability to these compounds to form complexes with metal cations, allowing them to act as chelators (Bharti et al., 2003). The mechanism of action of this class of molecule remains unclear, but it is thought to occur through multiple targets, such as cysteine proteases, present in various protozoa (Du et al., 2002; Greenbaum et al.,

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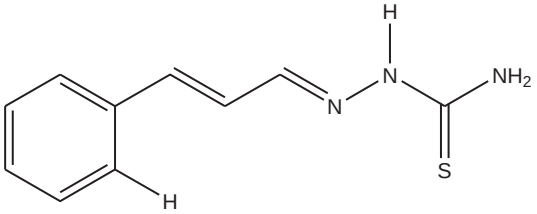
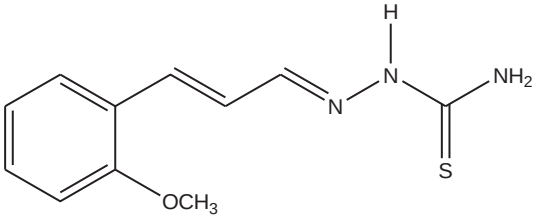
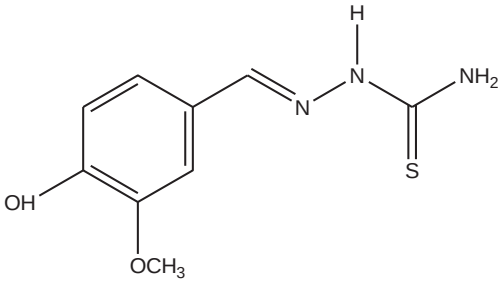
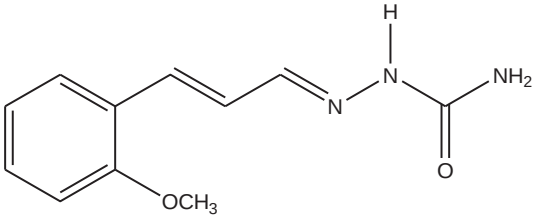
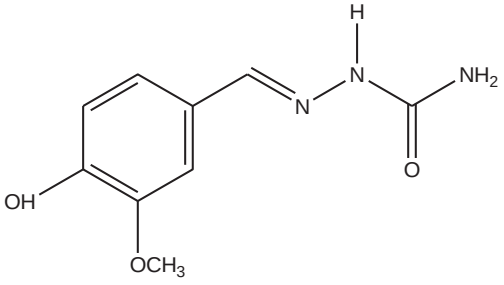
2004). Considerable attention has been focused on NO (nitric oxide) production, because of the crucial role that it plays as a cell signaling agent and its function as an antileishmanial effector molecule (Genestra et al., 2003a,b,c, 2006; Paveto et al., 1995). Furthermore, more recent results from our laboratory also reported the effect of drugs on the NO production by the *Leishmania amazonensis*, corroboring the above data (Soares-Bezerra et al., 2008).

As part of our research into chemotherapy to treat diseases caused by trypanosomatids, five thiosemicarbazones and semicarbazones were synthesized (Table 1) in an effort to obtain high trypanocidal activity with low toxicity. *In vitro* experiments using *T. cruzi* were carried out to evaluate the effect of these compounds against cultured trypomastigotes (from LLC-MK2 cells) and amastigotes lodged in both mouse and human macrophages. In addition, the *in vitro* toxicity of those derivatives was evaluated on murine macrophages. The enzymatic activity of the nitric oxide synthase (NOS) of the parasite was also evaluated, because this enzyme could be a potential target for these compounds.

Table 1
Inhibition of *T. cruzi* trypomastigote forms by thiosemicarbazones and semicarbazone derivatives (μM), toxicity to mice peritoneal macrophage (% cell death).

Inhibition of *Trypanosoma cruzi* trypomastigote forms/toxicity to macrophage			
Compound	Structure	LD_{50}/24 h μM	% cell death Toxicity
1		1.6	0.1
2		0.4	0.1
3		1.8	0.1
4		1.4	0.1
5		1.5	0.1

2. Materials and methods

2.1. Chemistry

The semicarbazone/thiosemicarbazone derivatives were synthesized as described in the literature (Heilbron et al., 1923; Oliveira et al., 2008). Benznidazole (Bz) (Rochagan – Roche) was obtained from the Instituto de Pesquisa Evandro Chagas IOC/FIOCRUZ, Rio de Janeiro, Brazil. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO, USA. Materials used were obtained from commercial supplies and used without purifications, unless otherwise noted. Melting points were determined with a Kofler (Jasco DIP-370) apparatus, and they have not been corrected. Using tetramethylsilane as the internal reference, ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-200 MHz instrument at room temperature. The general procedure for preparation of thiosemicarbazones and semicarbazones (**1**–**5**) was similar to that described in the literature (Heilbron et al., 1923; Oliveira et al., 2008). Briefly: a mixture of corresponding aldehydes (2 mmol) and thiosemicarbazide or semicarbazide (2 mmol) was dissolved in ethanol (10 mL) with a few drops of concentrated sulfuric acid for thiosemicarbazones or sodium acetate solution for semicarbazones. The resulting mixture was stirred at room temperature for 1 h or until the reaction finished, as observed by thin layer chromatography (TLC). The solvent was removed under reduced pressure, and the resulting solid was recrystallized from methanol to provide the thiosemicarbazones **1**–**3** and the semicarbazones **4**–**5**. The compounds were fully characterized as follows: 4-N-styryl-thiosemicarbazone (**1**): Yield 50%; m.p. 94–96 °C; ^{1}H NMR (DMSO-d_6) δ: 11.40, 8.18, 7.89, 7.52, 7.40, 7.28 and 6.88; ^{13}C NMR (DMSO-d_6) δ: 176.86, 144.26, 143.78, 138.11, 135.01, 128.07, 126.13 and 124.22. 4-N-(2′-methoxy styryl)-thiosemicarbazone (**2**): Yield 82%; m.p. 186–188 °C; ^{1}H NMR (DMSO-d_6) δ: 11.34, 8.14, 7.87, 7.57, 7.31, 7.17, 7.04, 6.98, 6.96 and 3.85; ^{13}C NMR (DMSO-d_6) δ: 177.08, 156.38, 145.34, 133.48, 129.78, 126.96, 125.27, 123.66, 120.26, 111.07 and 55.04. 4-N-(4′-hydroxy-3′-methoxybenzyl)-thiosemicarbazone (**3**): Yield 75%; m.p. 204–206 °C; ^{1}H NMR (DMSO-d_6) δ: 9.34, 9.09, 8.46, 7.38, 7.23, 6.91 and 3.77; ^{13}C NMR (DMSO-d_6) δ: 176.18, 147.22, 146.40, 132.70, 126.50, 123.46, 115.23, 111.02 and 55.54. 4-N-(2′-methoxy styryl)-semicarbazone (**4**): Yield 80%; m.p. 197 °C; ^{1}H NMR (DMSO-d_6) δ: 10.15, 8.32, 7.40, 7.12, 7.07, 7.04, 6.99, 6.96, 3.85 and 2.98; ^{13}C NMR (DMSO-d_6) δ: 156.17, 156.11, 142.23, 130.72, 129.30, 126.61, 125.85, 123.96, 120.25, 111.03 and 55.03. 4-N-(4′-hydroxy-3′-methoxybenzyl)-semicarbazone (**5**): Yield 76%; m.p. 216 °C; ^{1}H NMR (DMSO-d_6) δ: 10.28, 9.30, 7.27, 7.16, 7.12, 5.53, and 3.77; ^{13}C NMR (DMSO-d_6) δ: 156.78, 147.88, 145.32, 137.19, 121.11, 119.0, 118.13, 112.17 and 55.85.

2.2. Animals and parasites

Swiss mice (males, weighting 20–25 g) acquired from Centro de Criação de Animais de Laboratório (CECAL)-FIOCRUZ were used to obtain peritoneal macrophages for infection and isolation of *T. cruzi*. The experiments were conducted using a protocol approved by the Comitê de Ética no Uso de Animais (CEUA-FIOCRUZ, protocol number P0369-07). Trypomastigote forms of *T. cruzi* (Y strain) were maintained by animal passage and used to infect the LLC-MK2 cell lineage as previously described (Pinho et al., 2002). The parasites obtained from this infection were used in all further experiments.

2.3. Drug assays

The compounds were tested (thiosemicarbazones **1**–**3** and the semicarbazones **4** and **5**) in a concentration range of 5–320 μg/mL. The compounds were solubilized in dimethylsulfoxide (DMSO) with the final concentration of the solvent in the experiments never exceeding 1.6%, which is not hazardous to the host cells, then added to a 96 wells microplate and incubated at 37 °C for 24 h with *T. cruzi* trypomastigotes (obtained from LLC-MK2 cells, as described above) at a concentration of 4×10^6 cells/mL. The drug effects were analyzed by counting the remaining parasites and/or based on the results from the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) dye reduction assay as described by Mosmann (1983). Briefly, after the incubation time, 5 mg/mL MTT in PBS was added (22 μL/well) and the plates were further incubated for 4 h at 37 °C. The resulting formazan crystals formed were dissolved with DMSO (80 μL/well) and the samples were measured in a spectrophotometer at 490 nm. Although all thiosemicarbazone and semicarbazone derivates were used for the toxicity assays, only the most effective was used in the following experiments. Tests were performed in triplicate and Bz was used as a reference drug.

2.4. Cytotoxicity assay

The cytotoxic effect, expressed as the percentage of cell death, was assayed using mouse peritoneal macrophages. The cells were isolated from the peritoneal cavity of the animals in cold RPMI 1640 medium supplemented with 1 mM HEPES, Penicillin G (10^5 IU/L) and streptomycin sulfate (10 g/L). Cells (2×10^5 per-well) were cultivated at 37 °C in a humidified 5% CO_2 atmosphere. After 2 h of incubation, the non-adherent cells were washed out twice with RPMI. Test compounds and Bz were added in the highest concentration used in the activity test (320 μg/mL). Then, MTT was applied to the wells as described above. Tests to quantify the cytotoxicity on human macrophages were not performed due to the limited number of cells available from each donor.

2.5. Nitric oxide synthase (NOS) activity

The NOS activity of *T. cruzi* trypomastigotes from cell cultures in the absence (control) or presence of the most effective compound was determined by measuring the NADPH consumption at 340 nm because this cofactor is consumed during the conversion of L-arginine to L-citrulline and nitric oxide (NO) by NOS (Genestra et al., 2006). Briefly, the complete enzyme reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM $CaCl_2$, 0.1 mM NADPH, 80 μM H_4B, 10 μM FAD, 10 μM FMN, 0.1 mM L-arginine, parasite extract (16.2 μg/mL of protein) and the compound/Bz using a concentration corresponding to the *in vitro* LD_{50}/24 h in a final volume of 1 mL. The control group contained the components of the reaction mixture, except the drugs.

2.6. Growth inhibition of intracellular amastigotes

To evaluate the effect of the thiosemicarbazone **2**, two cellular preparations were used: (a) mouse macrophages were isolated from the peritoneal cavity of Swiss mice using cold RPMI 1640 medium, supplemented with 1 mM HEPES, Penicillin G (10^5 IU/L) and streptomycin sulfate (0.1 g/L); (b) human peripheral blood mononuclear cells (PBMC) from eight health donors were separated by Ficoll-Hypaque gradient centrifugation (Histopaque) from Buffy coat preparation, and the monocyte-derived macrophages were isolated by adherence. Briefly, 2×10^6 cells (mouse/human macrophages) were plated in Lab-Tek tissue chamber slides and maintained in supplemented RPMI at 37 °C, 5% CO_2. One hour later, the chambers were extensively washed with RPMI to remove non-adherent cells, and the remaining adherent cells were subsequently cultured in RPMI medium containing 10% fetal calf serum (FCS) during 24 h. After this time, the cultures were infected with

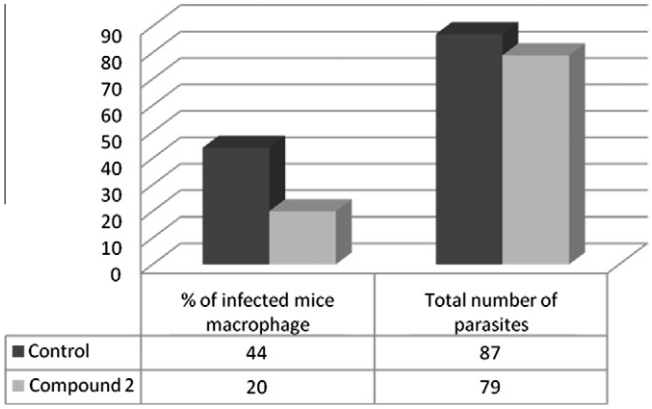


Fig. 1. Effect of (2-methoxy-styryl)-thiosemicarbazone on the infection of mouse peritoneal macrophages by *T. cruzi*. Mouse peritoneal macrophages infected with trypomastigote forms of *T. cruzi* (Y strain) and treated with (2-methoxy-styryl)-thiosemicarbazone (102.5 ± 0 μg/mL). Sensitivities of intracellular amastigotes were determined as MEM ± SD of triplicates of experimental groups. Control: Infected macrophage without treatment or DMSO.

2×10^7 trypomastigote forms of *T. cruzi* for 3 h at 37 °C, 5% CO_2 and the non-interiorized parasites were removed by washing the macrophages three times with RPMI. The chosen compound was solubilized in DMSO and added to the *T. cruzi* infected macrophage cultures for 24 h, using a concentration corresponding to the *in vitro* LD_{50}/24 h. Drug activity was determined as the percentage of the infected macrophages in drug-treated cultures compared to the infected control (without treatment or DMSO), using optical microscopy following methanol fixation and Giemsa staining. Quantification of intracellular amastigotes was determined as the MEM ± SD of three experiments (Alves et al., 2004; Bourguignon et al., 2009). The protocol for the use of human cells was approved by Comitê de Ética em Pesquisa (CEP/FIOCRUZ, No. 535/09).

2.7. Light microscopy of the human macrophage experiment

The standard images of infection (without treatment) and the morphology of human macrophages infected with *T. cruzi* and treated with 4-N-(2′-methoxy styryl)-thiosemicarbazone (**2**), were evaluated at 40× and 100× magnification using a digital system DSC-W30 Sony camera fitted with a zoom microscope-magnifying lens, which produced enhanced black and white images yielding image tiles of 6.0 megapixels, zoom 4.4 (Fig. 2).

2.8. Statistical analysis

Data were analyzed using Student's *t* test for significance. *P* values <0.05 were considered significant.

3. Results and discussion

Current treatment for Chagas' disease is primarily dependent on benznidazole (Bz), a 2-nitroimidazole drug, which is the only drug to treat Chagas' disease available for human use in Brazil and Argentina. This specific chemotherapy has limitations, such as a lack of effectiveness to achieving parasitological cure or in the prevention of the chronic phase of the disease, in addition to the emergence of parasite resistance (Pérez-Rebolledo et al., 2008). There is a considerable need for the development of new compounds to improve the chemotherapy of Chagas' disease.

Thiosemicarbazones and semicarbazones are an important class of compounds that have been shown to have several biological activities, including effects on pathogenic parasites. As a result, they have been extensively studied in medicinal chemistry (Beraldo and Gambino, 2004; Fabrino et al., 2004; Urbina, 1999). In the present work, five derivatives of thiosemicarbazones and semicarbazones were evaluated *in vitro* against culture trypomastigotes of *T. cruzi*. Among the compounds investigated, the compound 4-N-(2′-methoxy styryl)-thiosemicarbazone (**2**) was the most efficient with a LD_{50}/24 h value of 0.4 μM, while Bz showed a lower activity than the test compounds (1.8 μM). No macrophage toxicity was observed by any of the compounds, indicating that their activity was specific for the parasite forms investigated, while Bz presented toxicity under our conditions. Evaluating the results of activity, it appears that the addition of the methoxy group in the *ortho* position of the aromatic ring and the presence of sulfur are associated to an increase in the effectiveness of this thiosemicarbazone derivate against this parasite form. Comparing the similarity of the molecular structure of compounds **2** [4-N-(2′-methoxy styryl)-thiosemicarbazone] and **4** [(4-N-(2′-methoxy styryl)-semicarbazone], it appears that the change of sulfur to oxygen significantly reduced the trypanocidal activity of compound **4**, as the LD_{50} increased considerably. The presence of hydroxyl and methoxy groups in *meta* and *para* positions, respectively, and the absence of the styryl moiety in compounds **3** and **5** do not improve the biological activity, that is, they are not advantageous. In a comparative analysis of data from our group, when thiosemicarbazones and semicarbazones were assayed against the blood stream forms of *T. cruzi* and *L. amazonensis* promastigotes, a potent increase of the activity was observed under specific conditions for each parasite form. In addition, these compounds were more potent than pentamidine and Bz, drugs used for the treatment of leishmaniasis and Chagas' disease, respectively (Soares, 2007). Furthermore, a study comparing the effectiveness of compound **2** in intracellular amastigotes using mouse peritoneal and human macrophages was performed. It is important to note that, in general, during the evaluation of the *in vitro* drug activity, the protocols were always performed using infected murine peritoneal macrophage because they are very easy to obtain and manipulate. Our results indicate that this thiosemicarbazone has a potent activity against intracellular

Table 2
Effect of (2-methoxy-styryl)-thiosemicarbazone on the infection of human macrophages by *T. cruzi*. Human macrophages infected with trypomastigote forms of *T. cruzi* (Y strain) and treated with (2-methoxy-styryl)-thiosemicarbazone (0.4 μM). Sensitivities of intracellular amastigotes were determined as MEM ± SD of triplicates of each assay. Control: Infected macrophages without treatment or DMSO.

Human donors	% of infected human macrophage ± SD			No. of amastigote/human macrophage ± SD		
	Control	Thiosemicarbazone	% of reduction	Control	Thiosemicarbazone	% of reduction
1	48 ± 2.00	1.0 ± 1.70	97	3.5 ± 0.07	0.4 ± 0.75	88
2	66 ± 0.00	1,7 ± 1.50	96	3.8 ± 0.06	0.9 ± 0.81	76
3	38 ± 5.30	10.0 ± 2.50	72	5.7 ± 0.12	2.6 ± 0,32	55
4	48 ± 12.2	14.3 ± 0.60	70	2.4 ± 0.10	2.0 ± 0.10	17
5	10 ± 2.90	0.7 ± 0.60	94	1.5 ± 0.20	1.0 ± 1.00	23
6	11 ± 1.00	0.0 ± 0.00	100	1.5 ± 0.06	0.0 ± 0.00	100
7	27 ± 6.00	3.0 ± 1.00	73	1.5 ± 0.29	0.7 ± 0.25	59
8	08 ± 1.20	2.0 ± 1.00	79	2.0 ± 0.10	1.0 ± 0.00	50

amastigote forms of *T. cruzi* in both mouse peritoneal and human macrophages.

Assays using mouse macrophages infected with *T. cruzi* showed that the treatment for 24 h with 0.4 μM of 4-N-(2′-methoxy styryl)-thiosemicarbazone (**2**) resulted in a 54.5% reduction in the mean percentage of infected macrophages and a 9.2% decrease of intracellular parasites (Fig. 1).

The role of human macrophages is expected to vary since they came for different individuals. Also, it is important to notice that the differentiation of monocytes to macrophages that occurred *in vitro*, would lead to changes in the cell behavior. These modifications could be responsible for a potential variation in the infection rate (de Souza et al., 2010) and in the parasite's vulnerability within the cell. In fact, even under the effect of all those different situations, the effectiveness of the compounds was much more evident in assays conducted with infected human macrophages, with a higher reduction in the percentage of the infected (70% in sample number 4 to 100% in sample number 6). Infected macrophages of donors 1, 2 and 5 showed excellent response to treatment, with reductions of 97%, 96% and 94% in the number of infected host cells, respectively (Table 2). Also, a strong reduction in the percentage of amastigote forms lodged in human macrophages was observed in most of the studied samples. In this case, the percentage of trypanocidal activity of the compound against amastigote forms was higher in human macrophages (samples 1, 2, 3, 6, 7 and 8), showing a decrease in the amount of parasites in a range of 50–100%. However, samples 4 and 5 showed a considerable decrease in the number of infected macrophages (70% and 94%), while the decrease of the number of parasites within macrophages was not significant, as expected (Table 2).

There is a diverse range of molecular mechanisms through which parasites can invade the host cell that may correspond to differences in the available receptors on the surface of each specific cell type (Gros et al., 2006). Also, it has been show that some surface molecules in both mouse and human macrophages are proteins that are structurally, functionally and antigenically different (Saraiva et al., 2007). The differences observed in our experiments comparing mouse and human macrophages could be related to these observations. Our results show that this thiosemicarbazone derivate was more potent against parasites within human cells, which in some way mimic the *in vivo* situation more accurately than mouse peritoneal macrophages. The evaluation of the effect of 4-N-(2′-methoxy styryl)-thiosemicarbazone (**2**) against amastigotes of *T. cruzi* lodged in human macrophages using light microscopy showed a complete elimination of the parasites, without any hazardous effect to the macrophage of donor 1 (Fig. 2).

Data from literature demonstrated that trypanosomatids are able to produce nitric oxide, through a constitutive nitric oxide synthase (cNOS) that allows the parasites to survive within macrophages, participating in the host–parasite interaction (Basu et al., 1997; Géigel and Leon, 2003; Genestra et al., 2003a,b; Paveto et al., 1995; Pereira et al., 1997). In a search for a potential target for compound **2**, an assay was carried out to evaluate the effect of this compound on the *T. cruzi*-NOS activity. The NOS activity

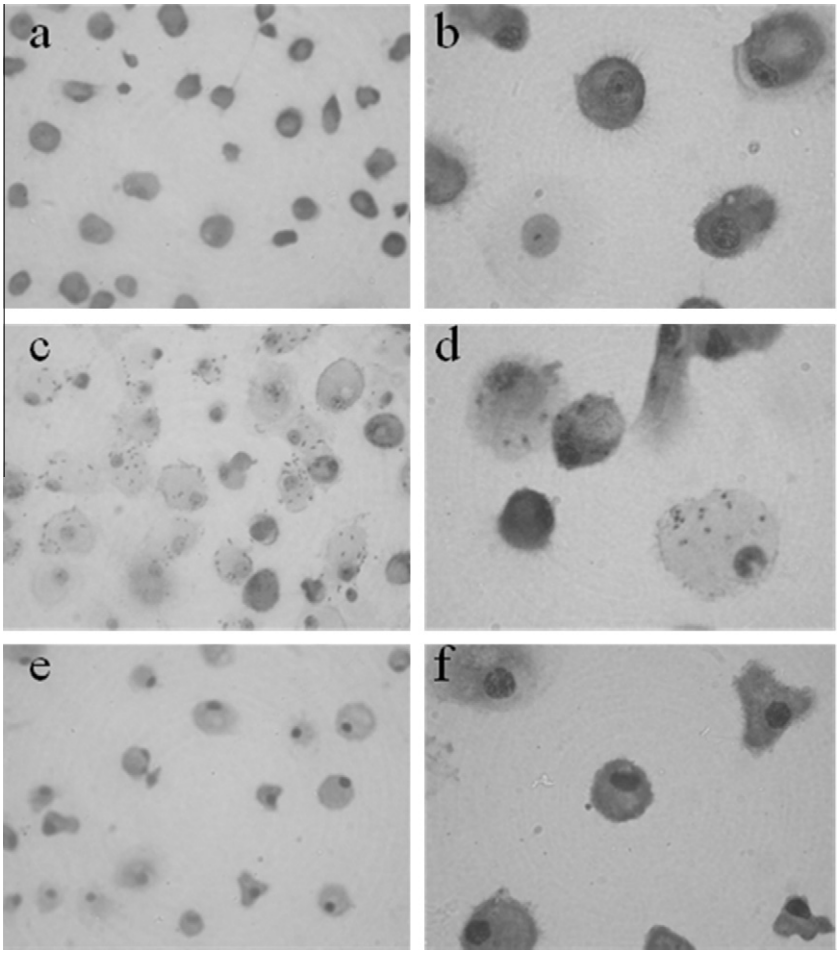


Fig. 2. A light microscopy view of the effect of 4-N-(2′-methoxy styryl)-thiosemicarbazone (**2**) against amastigotes of *T. cruzi* lodge in human macrophages (donor 1). (a, b) Uninfected macrophages, (c, d) infected macrophages and (e, f) infected and treated with compound **2**. Magnification 40× (a, c and e) and 100× (b, d and f).

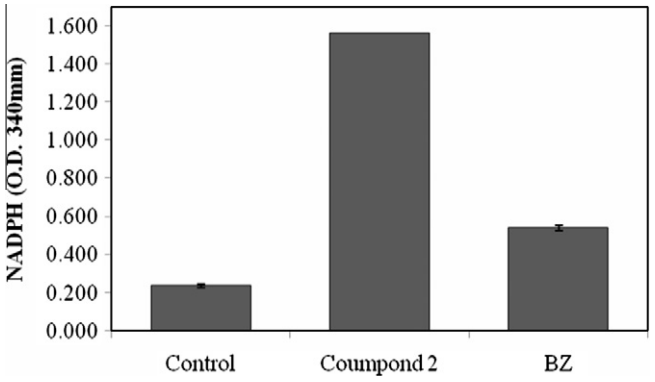


Fig. 3. Evaluation of the activity of NOS from *T. cruzi* trypomastigotes, according to the consumption of NADPH, in absence (control)/presence of 4-N-(2′-methoxy styryl)-thiosemicarbazone (**2**) and Bz. Data represent MEM ± SD of three independent determinations.

was assayed on the extracts of *T. cruzi* cell culture trypomastigotes in the absence and presence of compound **2** and Bz. The results showed that this compound and Bz were able to inhibit the enzyme activity approximately 6.5- and 2-fold, respectively, as demonstrated by the decrease in NADPH consumption (Fig. 3).

These preliminary data allow us to suggest that the killing of *T. cruzi*, as both intracellular amastigotes and culture cell trypomastigotes, would be associated to reduction of the parasite NOS activity and not to the NO produced by the macrophages, which means that this radical can not be considered a killer molecule. It has been considered by several authors (Chen and Rosazza, 1994; Fabrino et al., 2004; Paveto et al., 1995) that the parasite NOS activity and its capacity to produce NO, is a defense mechanism of trypanosomatids. In this study, it was demonstrated that parasites loose that capacity in the presence of the tested compound, and as a probable consequence, became more sensitive to the toxic molecules produced by macrophages.

In conclusion, this class of molecules presents high stability in different conditions (Beraldo, 2004), and no significant toxicity to the host cells, encouraging us to continue the present investigations through both *in vitro* and *in vivo* experiments. Also, it is important to carry out further studies of the metabolic pathways occurring within this parasite, in order to define more precisely the potential target for these compounds, as suggested by us.

Acknowledgments

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